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Eukaryotic membrane protein overproduction in *Lactococcus lactis*

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Eukaryotic membrane proteins play many vital roles in the cell and are important drug targets. Approximately 25% of all genes identified in the genome are known to encode membrane proteins, but the vast majority have no assigned function. Although the generation of structures of soluble proteins has entered the high-throughput stage, for eukaryotic membrane proteins only a dozen high-resolution structures have been obtained so far. One major bottleneck for the functional and structural characterisation of membrane proteins is the overproduction of biologically active material. Recent advances in the development of the *Lactococcus lactis* expression system have opened the way for the high-throughput functional expression of eukaryotic membrane proteins.

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Introduction

Membrane proteins fulfil many essential functions in the eukaryotic cell. The flow of ions, nutrients and communication signals across the membrane is facilitated by ion channels, transporters and receptors. Other membrane proteins, such as photosystems, the complexes of the respiratory chain and ATP synthase, are essential for the conversion of energy. Large membrane protein complexes such as the translocon, the nuclear pore and the translocases of the inner and outer mitochondrial membrane (TIM/TOM complexes) also play a key role, mediating the pathways for sorting, translocation and insertion of proteins. Membrane proteins have been implicated in many diseases, because they are positioned at the apex of signalling pathways that regulate cellular processes. This central role makes them a good target for drug treatment and G-protein-coupled receptors (GPCRs), ion channels and neurotransmitter transporters currently constitute more than 40% of all established drug targets [1].

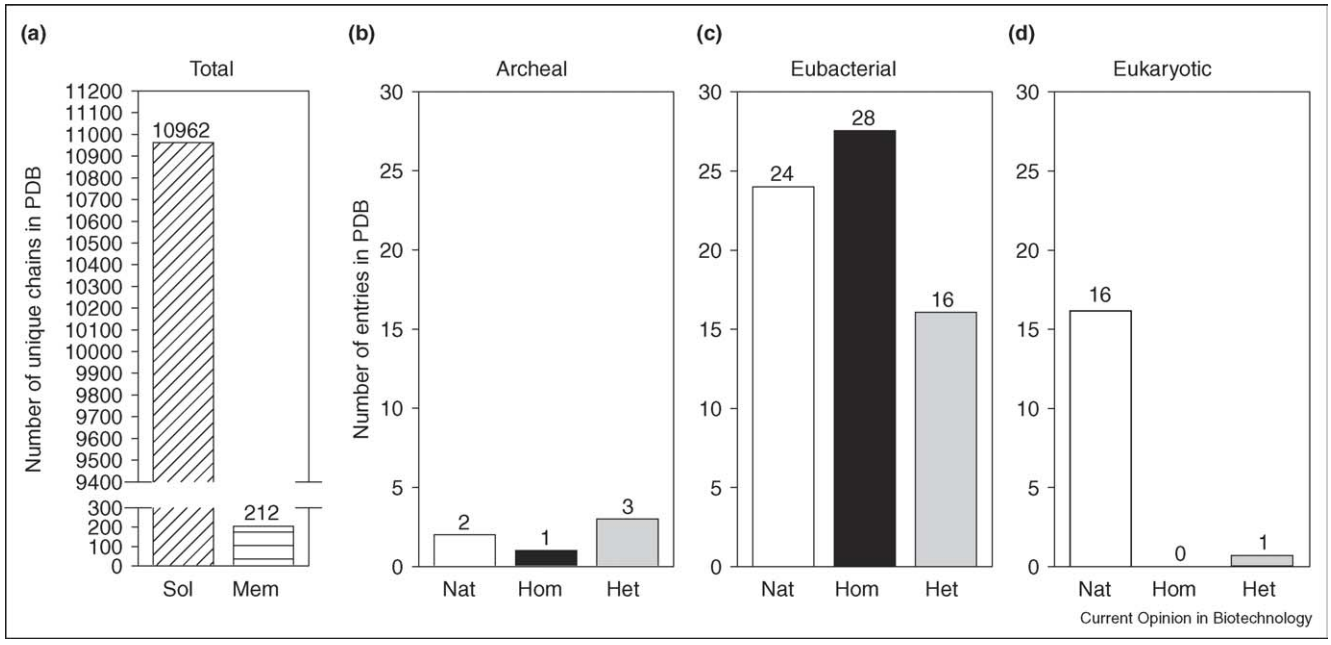
Genome sequencing projects have identified vast numbers of novel open reading frames that contain predicted transmembrane segments. The division of these unknown membrane proteins into families is relatively straightforward, but the assignment of function is not because the structural basis of ligand or substrate recognition is often unknown. If one considers polypeptide chains with less than 90% sequence identity, there are 50 times more known structures of soluble proteins than membrane proteins (Figure 1). Structures of eubacterial membrane proteins constitute 80% of the entries and were obtained from material that was either naturally abundant or homologously expressed. The structures of only 17 eukaryotic integral membrane proteins have been determined. In 16 of these cases, the protein under study was purified from abundant natural sources (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). The only exception is the crystal structure of the rat voltage-dependent shaker family potassium channel, which was obtained using protein expressed in *Pichia pastoris* (see Update).

To increase our knowledge of the function and structure of eukaryotic membrane proteins, novel approaches for their over-production need to be developed. This review provides a short description of the published expression strategies and goes on to discuss the recently developed *Lactococcus lactis* expression system.

Eukaryotic expression systems

The advantages of eukaryotic hosts for the expression of eukaryotic membrane proteins are clear. The systems for translation, targeting, insertion and post-translational modifications are compatible, thus the proteins are likely to be well-folded and active. Microbial hosts like *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* or *Pichia pastoris* have the added advantage of being cheap and easy to culture in large amounts. Among the disadvantages of these systems are the differences in post-translational modifications and the potential proteolysis of the target protein by endogenous proteases. Heterologous expression has been improved by taking advantage of yeast

Figure 1



Numbers of high-resolution structures of soluble proteins and membrane proteins. **(a)** The total number of polypeptide structures with less than 90% sequence identity for soluble proteins (diagonal stripes) and membrane proteins (horizontal stripes) deposited in the Protein Data Bank (PDB) by August 2005. The number of PDB entries of **(b)** archeal, **(c)** eubacterial and **(d)** eukaryotic integral membrane proteins divided into three categories indicating the source of the material used for the crystallization trials: naturally abundant (Nat, white), homologously expressed (Hom, black) and heterologously expressed (Het, grey).

physiology, such as tuning of the expression levels to avoid the unfolded protein response (a signaling mechanism triggered by the stress of protein expression) [2]. It is also possible to tailor metabolism so that the survival of the host is dependent on the functional expression of the protein [3,4]. Modification of the expressed protein through the use of fusion partners can also be advantageous; for example, C-terminal fusion proteins have been used to stabilize the expression of GPCRs, whereas the addition of N-terminal signal peptides can improve targeting and insertion [5–7].

Insect cell expression systems have been used for the successful overproduction of functional GPCRs under the control of baculovirus promoters [8] or endogenous promoters in transgenic insects [9]. Using these systems post-translational acylation of the receptors, which is important for their regulation, is achieved, but compatible N-glycosylation is not [10]. GPCRs, ion channels and transporters have been functionally expressed in mammalian cell lines that have compatible systems for post-translational modifications, targeting, insertion and folding [11•,12,13]; however, these systems are technically challenging and relatively expensive.

Bacterial expression systems

Bacterial expression systems are convenient and relatively cheap. However, potential difficulties arise from differences in membrane protein biogenesis, such as the transcription and translation machinery, membrane composition, and the targeting, insertion and folding pathways [14••]. The inability of bacterial expression systems to introduce post-translational modifications, such as glycosylation, could affect function, although this can also be an advantage for crystallization trials.

The Gram-negative bacterium *Escherichia coli* has been the main host for the production of soluble proteins and prokaryotic membrane proteins. High-level expression of functional eukaryotic membrane proteins has also been achieved in *E. coli*, but in most cases problems such as low-level expression, toxicity and inclusion body formation tend to occur. These problems can be overcome to some extent by changing media components, carbon source or the growth temperature [15]. The selection of strains with improved production properties [16] or the use of strains in which proteases have been deleted has also been successful [15]. Likewise, construct-optimisation — through the use of different promoters, fusion partners [17–19], truncations and the addition of N-terminal signal peptides — has proved beneficial [20]. Methods for the high-throughput screening of endogenous membrane protein overexpression have been developed, but have not been applied to eukaryotic membrane protein production in *E. coli* [21,22]. In another approach, the archaebacterium *Halobacterium salinarium* was used to

express eukaryotic membrane proteins as fusion partners of bacterio-opsin [23].

The lactococcal expression system

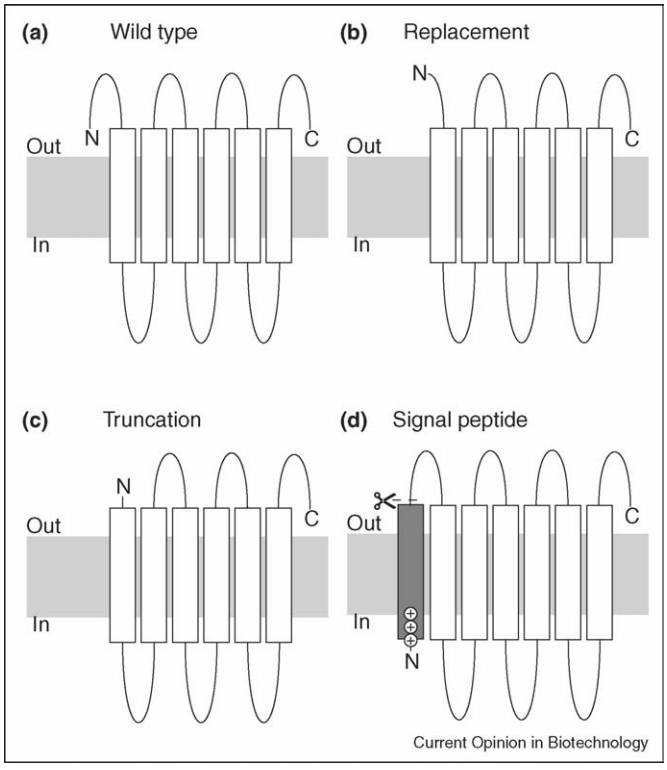
The use of the Gram-positive bacterium *L. lactis* as an expression system has great potential for the overproduction of membrane proteins and has been used successfully for the homologous overexpression of transporters from the major facilitator superfamily, ABC transporters, mechanosensitive channels and peptide transporters [24••] Heterologous expression of eukaryotic membrane proteins in *L. lactis* was first demonstrated with the human KDEL receptor, the hydrogenosomal ADP/ATP carrier and yeast mitochondrial carriers, which were all expressed in a functional form and at high levels [24••]. A human ABC transporter has also been overproduced in a functional form in this host [25•].

Several properties of *L. lactis* make it ideal for the overproduction of membrane proteins for functional and structural studies, as discussed below. The lactic acid bacterium grows to high cell densities (about 1 h doubling time) without the need for aeration, and its single membrane simplifies membrane protein targeting, insertion and cell fractionation. The nisin-inducible expression system [26] was developed in this host and offers a strong and tightly regulated promoter system that allows highly reproducible expression even when the proteins are toxic to the cell [24••]. In all reported cases where *L. lactis* has been used for expression, a specific transport or binding activity of the protein was observed, demonstrating that the system is reliable and reproducible for the production of functional material. Activities could be determined even when the expressed protein was barely detectable by western blotting. Furthermore, the expressed proteins were only found in the membrane and not in inclusion bodies, which meant that their activities could be established without the need for purification, refolding and reconstitution. This important property enables functional characterisation in whole cells, because substrates, ligands or inhibitors can pass through the cell wall to interact directly with the membrane protein. Studies to identify the transporter substrates and receptor ligands are facilitated because *L. lactis* usually lacks endogenous proteins with the same function. So far, significant degradation of isolated membrane proteins has not been observed, because the bacterium has a relatively mild proteolytic capability. Of note for structural studies, the availability of multiple amino acid auxotrophic strains of *L. lactis* has facilitated the incorporation of selenomethionine for the phasing of X-ray diffraction data and for the specific labelling of proteins for NMR. Taken together, these properties clearly highlight the importance of *L. lactis* as a suitable host for the overexpression of membrane proteins.

Recently, we have employed this system for the expression and functional characterisation of members of the mitochondrial carrier family, which are found exclusively in eukaryotes [24••]. Mitochondrial carriers carry metabolites and cofactors through the mitochondrial inner membrane and have a unique biogenesis pathway (see [27,28] for recent reviews). When the carriers are overexpressed in *E. coli*, they are produced in inclusion bodies from which they can be purified, refolded and reconstituted, but the efficiency is low [28–30]. When mitochondrial carriers are expressed in yeast, degradation is observed during the isolation of mitochondria using standard procedures. Clearly both these hosts have disadvantages, but mitochondrial carriers have been overexpressed successfully in *L. lactis* in the cytoplasmic membrane. The observed expression levels are sufficient to enable crystallisation trials to be performed for structural studies [24••]. Transport assays can be carried out in whole cells if sufficient amounts of exchangeable substrate are present in the cytosol. Alternatively, it is possible to isolate lactococcal membranes by mechanical disruption and differential centrifugation, to fuse them to liposomes by freeze-thawing and extrusion, and to load them with cold substrate before carrying out the transport assay.

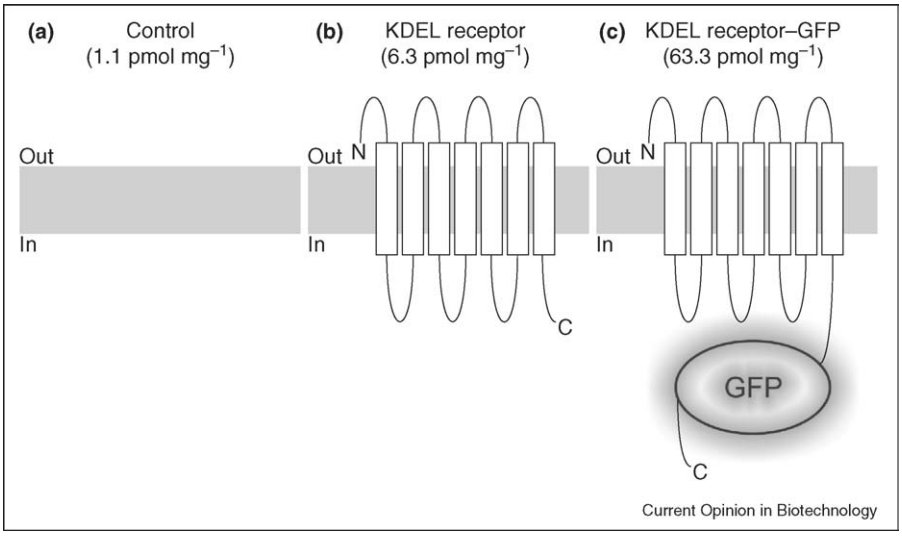
Expression trials have revealed possible limitations of the expression system. The yeast ADP/ATP carrier AAC2 is expressed poorly, whereas another isoform, AAC3, is expressed well, even though they share 87% identity (ERS Kunji *et al.* unpublished). The only major difference between the two proteins is in the N-terminal region preceding the first transmembrane α-helix. Inhibitor studies have shown that AAC3 is expressed in the lactococcal membrane with its N and C terminus to the outside of the cell [24••] in accordance with the 'positive inside rule' [31]. The requirement to translocate the N terminus to the outside of the cell could have been a limitation in the insertion of the carrier. An N terminus-out topology is a potential impediment in expression, as polytopic membrane proteins in bacteria usually have a cytoplasmic location for both the N and C termini (57%) [32]. This problem could be resolved by modifying the N terminus or through the introduction of a signal peptide for the translocation of the N-terminal region to the outside of the cell (Figure 2), as has been done in other expression systems [5–7,20]. Another potential limitation of the lactococcal expression system is the AT-rich codon usage of the organism (65%). Codon optimisation may be a general necessity to improve the expression of mammalian proteins in this organism [33]. Green fluorescent protein (GFP) has been used successfully in *E. coli* as a quantifiable marker for the overexpression of endogenous membrane proteins [21] and for the localisation of the C terminus in a high-throughput fashion [32]. Recently, the functional expression of the human KDEL receptor in *L. lactis* was improved more than ten times by using a GFP fusion to the C terminus (Figure 3) [34]. The successful use of GFP opens possibilities for the convenient detec-

Figure 2



Schematic representations of the yeast mitochondrial ADP/ATP carrier AAC2 as **(a)** wild-type protein, **(b)** with an AAC3 N-terminal replacement, **(c)** as an N-terminal truncation or **(d)** as a signal peptide fusion.

Figure 3



Functional overexpression of the human KDEL receptor as a wild-type protein and as a C-terminal GFP fusion protein. The numbers show the specific binding of the ligand [^{3}H]-YTSEHDEL to **(a)** isolated membranes of the control strain, **(b)** the strain expressing the wild-type KDEL receptor, and **(c)** the strain expressing the KDEL receptor–GFP fusion. (Data taken from [34].)

tion of membrane proteins in high-throughput expression studies.

Recently, the *L. lactis* expression system was used successfully to identify a novel adenine nucleotide transporter from the mitosome of *Entamoeba histolytica* [35••]. The substrate specificity, as well as the driving forces for transport, was determined in lactococcal membranes that contained the overexpressed transporter without the need for further purification. These results suggest that this expression system has great potential for the systematic assignment of protein function in high-throughput applications.

Conclusions

The expression studies described here prove the concept of using *L. lactis* for the overproduction of eukaryotic membrane proteins for functional and structural analysis. The expression system has many practical advantages, but it is also clear that difficult topologies and codon usage might limit expression. These problems can be resolved through optimisation of the expression constructs, showing that there is scope for further improvement of the system.

Although the lactococcal expression system has only been tried with a limited set of membrane proteins, there is no reason to assume that these approaches cannot be applied to other important eukaryotic membrane proteins. High-throughput protocols for the identification of orphan membrane proteins and structural genomics are currently being developed.

Update

The paper by Long, Campbell and Mackinnon presents the first structure of a eukaryotic integral membrane protein that was obtained by heterologous overexpression [36••]. The procedures for the expression of the voltage-dependent K^+ channel in *P. pastoris* were developed by Parcej and Eckhardt-Strelau [37].

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